

## Molecular analysis of the N gene of canine distemper virus in dogs in Brazil

[Análise Molecular do Gene N do Vírus da Cinomose em Cães do Brasil]

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### ABSTRACT

Eleven central-nervous-system samples collected from stray dogs between 2000 and 2004 were found positive by RT-PCR, which amplified a 480bp fragment of the N gene of canine distemper virus (CDV). Phylogenetic analysis based on partial N-gene sequences showed four major clusters. All dog strains segregated into cluster I, with a mean nucleotide identity of 95.8% and 95.6% with the Onderstepoort and Lederle vaccine strains, respectively. Cluster II contained all the raccoon-related strains, cluster III Orient strains and Cluster IV the Onderstepoort and Lederle vaccine strains, with a mean nucleotide identity of 99.7% between them. This is the first report of phylogenetic analysis of CDV strains in Brazil.

Keywords: dog, canine distemper virus, RT-PCR, phylogeny

### RESUMO

Onze amostras de sistema nervoso central de cães coletados entre 2000 e 2004 foram positivas pela RT-PCR, a qual amplificou um fragmento de 480pb do gene N do vírus da cinomose canina (VCC). A análise filogenética baseada na sequência parcial do gene N mostrou quatro principais agrupamentos genéticos. Todas as amostras de cães segregaram no agrupamento I, com identidade média de nucleotídeos de 95,8% e 95,6% com as amostras vacinais Onderstepoort e Lederle, respectivamente. O agrupamento II agregou todas as amostras relacionadas aos guaxinins. O agrupamento III agregou amostras orientais e o agrupamento IV agregou as amostras vacinais Onderstepoort e Lederle, com identidade média de nucleotídeos de 99,7% entre elas. Este é o primeiro relato de análise filogenética de amostras de VCC no Brasil.

Palavras-chave: cão, vírus da cinomose canina, RT-PCR, filogenia

### INTRODUCTION

Canine distemper virus (CDV) is a highly contagious pathogen that occurs worldwide and causes a fatal disease called canine distemper in carnivores (Scagliarini et al., 2003). The fatality rate of the disease is second only to that of rabies (Deem et al., 2000). CDV is a member of the genus *Morbillivirus* in the family

*Paramixoviridae* and causes generalized infection with prominent respiratory, gastrointestinal and nervous signs (Scagliarini et al., 2003).

The disease has been controlled by the use of attenuated live-virus vaccines, but a number of typical CDV-infected dogs have been observed in vaccinated dog populations worldwide

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(Blixenkronne-Moller et al., 1993; Gemma et al., 1996). These findings suggest that there are antigenic differences in some geographically distinct wild-type CDV and that a vaccine virus strain may be responsible for the resurgence of the disease (Harder and Osterhaus, 1997). Epidemiological and serological studies suggest that these "new" geographic wild-types have altered antigenicities compared with the same "old" geographic wild-type (Yoshida et al., 1998).

Genetic sequencing and subsequent phylogenetic analysis are methods that not only indicate the evolutionary relationship between samples collected in different geographical regions but also help to understand the antigenic differences between different biological samples. Nucleic-acid sequencing reveals the nucleotide sequence present in the gene being studied and thus makes it possible to predict the structure and function of the protein coded by the gene. When such studies are carried out with samples of pathogens, such as CDV, or even with vaccine strains, the results can help to control a particular pathogenic agent (Bellini et al., 1986; Sharma et al., 1992).

RNA viruses have high replication and mutation rates, and the latter are the primary source of genetic variations (Tsimring et al., 1996). The virus' ecological environment consists of basically two components: the internal one, represented by the cell itself, and the external one, represented by the uniqueness of the host within the ecological environment. These two components modulate the adaptive responses of the RNA viruses (Domingo and Holland, 1997). The mechanisms that are involved in fixing or removing a genetic mutation are the same ones that act in other living creatures, namely, natural selection, genetic drift (Felsenstein, 1971; Haigh, 1978) and population bottlenecks (Clarke et al., 1993). The different mutations that occur in virus populations are selected at random, and because of geographical barriers, these mutations become restricted to specific regions. All the mechanisms that cause genetic variations produce virus populations that can be identified according to their geographical origin (Clarke et al., 1993).

In Brazil, CDV has been diagnosed by histopathology and fluorescent antibody test (FAT) (Silva et al., 1999; Headly and Graça, 2000). Nonetheless, developments in molecular

techniques revealed the suitability of methods based on the nucleocapsid protein (N) gene (Frisk et al., 1999; Kim et al., 2001; Shin et al., 2004; Jóźwik and Frymus, 2005) for CDV detection as well as for molecular epidemiology studies (Scagliarini et al., 2003; Keawcharoen et al., 2005).

The N nucleoprotein is the most abundant structural viral protein and has been shown to possess regulatory functions such as transcription and replication, as well as encapsidation of the RNA genome into an RNase-resistant nucleocapsid (Stettler and Zurbriggen, 1995). Some variation of the N gene among CDV isolates has been demonstrated, despite the fact that this protein makes up the conserved region of the CDV genome (Parks et al., 1992; Curran et al., 1993).

The objective of the present study was to assess the molecular genetic diversity of Brazilian strains of CDV by analysis of partial nucleotide sequences of the most conserved region of the N gene using the reverse transcriptase polymerase chain reaction (RT-PCR) method developed for CDV detection.

## **MATERIALS AND METHODS**

The study used central nervous system (CNS) samples from 11 stray dogs from 10 municipalities in the state of São Paulo, southeastern Brazil (Table 1), collected between 2000 and 2004 and sent without any information on clinical signs to the Pasteur Institute as part of the Rabies Epidemiological Surveillance Program. These samples had previously been found positive by Sellers' staining (Tierkel and Atanasiu, 1996).

The Lederle/VR128 strain, which was kindly provided by Laboratório Biovet, was grown in Vero cells and used as a positive control for the RT-PCR.

A pair of primers was designed with Primer Premier 5.0<sup>®</sup> targeted to a highly conserved region of the 5' half of the CDV N gene with sequences retrieved from GenBank. The pair of primers in positions 654-670 and 1118-1133 of the Onderstepoort-vaccine-strain N gene, with a predicted amplicon of 480bp, were called CDV-

forward (5'-ACTGCTCCTGATACTGC-3') and CDV-reverse (5'-TTCAACACCAAC(T/C)CCC-3'), respectively.

Total RNA was extracted from CNS samples, Lederle strain (positive control) and ultra-pure water (negative control) with TRIzol<sup>1</sup>, according to the manufacturer's instructions. Next, 5µl of extracted RNA were added to the reverse-transcription (c-DNA synthesis) mix containing 1x First Strand Buffer<sup>1</sup>, 40U RNaseOUT<sup>1</sup>, 1.3mM of each dNTP, 50pmols of each primer, 8.5mM DTT, 200U Superscript II Reverse Transcriptase<sup>1</sup>, and RNase/DNase-free water to a final volume of 47µl and submitted to 42°C for 60min.

For the PCR amplification, 10µl of cDNA were added to a mix containing 1x PCR Buffer<sup>1</sup>, 50pmols of each primer, 0.2mM dNTP, 2.4mM MgCl<sub>2</sub>, 2.5U/µl Taq DNA polymerase Recombinant<sup>1</sup>, and RNase/DNase-free water to a final volume of 102 µl and submitted to an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 45s, 48°C for 45s and 72°C for 90s, and a final cycle for extension of 72°C for 10min. The PCR products were analyzed by electrophoresis on 1% agarose gel stained with ethidium bromide.

The amplified DNA fragments were purified with GFX PCR DNA and Gel Band Purification Kit<sup>2</sup> and submitted to bi-directional sequencing with DYEnamic ET Dye Terminator<sup>2</sup> according to the manufacturer's instructions; the sequences were resolved in a MegaBACE DNA sequencer<sup>2</sup>.

The final sequence of each sample, 447bp long, was aligned by the CLUSTAL/W method with Bioedit software (Hall, 1999) with homologous sequences retrieved from GenBank (Table 1) and the alignment used to build a Neighbor-Joining distance tree with the Kimura 2-parameter model and 1,000 bootstrap replicates with Mega 2.1 (Kumar et al., 2001).

## RESULTS

All of the 11 CNS samples collected from Brazilian dogs, which had previously been found

positive by Sellers' staining, were also positive for canine distemper virus by RT-PCR, as was the Lederle vaccine strain. No amplification was found in the negative control and no extra band was detected in any of the reactions (data not shown).

Phylogenetic analysis showed that the sequences of the 11 Brazilian dog strains produced a single cluster (cluster I) (Fig. 1) with an average identity (ID) of 99.2%. The Onderstepoort and Lederle vaccine strains segregated into the same cluster (cluster IV, ID= 99.7%). The ID of clusters I and IV (I x IV) was 95.9%, and the ID of cluster I with the Onderstepoort strain was 95.8% and with the Lederle strain 95.6%. Cluster II (raccoon strains, ID= 99.6%) had a 99.4% identity with cluster I (I x II), and cluster III (Orient strains<sup>3</sup>, ID= 99.6%) had a 98.8% identity with cluster I (I x III). The German dog strain 25/44-Hans segregated into cluster I with a 99.2% identity with the other samples in cluster I.

Furthermore, as seen in Fig. 1, cluster I is subdivided into clusters IA and IB, supported by bootstrap values of 83 and 58, respectively, with an ID of 99.3% between them. All the previous results are summarized in Table 2.

## DISCUSSION

A growing knowledge of molecular biology has led to the development of several N gene-based RT-PCR methods for the detection of CDV (Frisk et al., 1999). However, RT-PCR combined with nested PCR has also been used to detect CDV RNA in dogs and has been shown to be a more sensitive method (Kim et al., 2001; Shin et al., 2004; Józwick and Frymus, 2005).

In the present study, a practical RT-PCR protocol was developed to detect the CDV N gene in CNS samples from carnivores and to allow studies of the molecular diversity of the Brazilian CDV isolates detected. This new protocol does not use the nested step, thus simplifying the procedures and avoiding cross-contamination with amplicons between different samples.

<sup>1</sup>Invitrogen™, USA.

<sup>2</sup>Amersham Biosciences™.

<sup>3</sup>Strains that originated in China and Taiwan.

*Molecular analysis of the N gene...*

Table 1. Strain, accession number, detection and location of CDV strains used for phylogenetic analysis in this study.

Strain	Accession number	Detection (year/host)	Location(country/state)
IP1407	AY738624	2004/dog	Brazil/São Paulo
IP1682	DQ005126	2001/dog	Brazil/São Paulo
IP2376	DQ005131	2000/dog	Brazil/São Paulo
IP2392	DQ005127	2004/dog	Brazil/São Paulo
IP2397	AY738653	2004/dog	Brazil/São Paulo
IP2705	DQ005132	2001/dog	Brazil/São Paulo
IP3045	DQ005128	2004/dog	Brazil/São Paulo
IP3258	DQ005133	2004/dog	Brazil/São Paulo
IP3288	DQ005129	2004/dog	Brazil/São Paulo
IP3683	DQ005134	2004/dog	Brazil/São Paulo
IP4712	DQ005130	2004/dog	Brazil/São Paulo
2544/Hans	AJ009656	NI/dog	Germany/NI
98-2654	AY466011	1998/raccoon	USA/Illinois
98-2645	AY445077	1998/raccoon	USA/Illinois
01-2689	AY649446	2001/raccoon	USA/Illinois
00-2601	AY443350	2000/raccoon	USA/Illinois
98-2646	AY542312	1998/raccoon	USA/Illinois
Onderstepoort	AF305419	Vaccine strain	NI
Lederle	AY738625	Vaccine strain	NI
Kaohsiung	DQ522030	2006/NI	Taiwan/NI
Pingtung	DQ435615	2006//NI	Taiwan/NI
TN-N	AY390348	2003/NI	China/NI
A75/17	AF164967	NI/NI	NI

NI = No information available

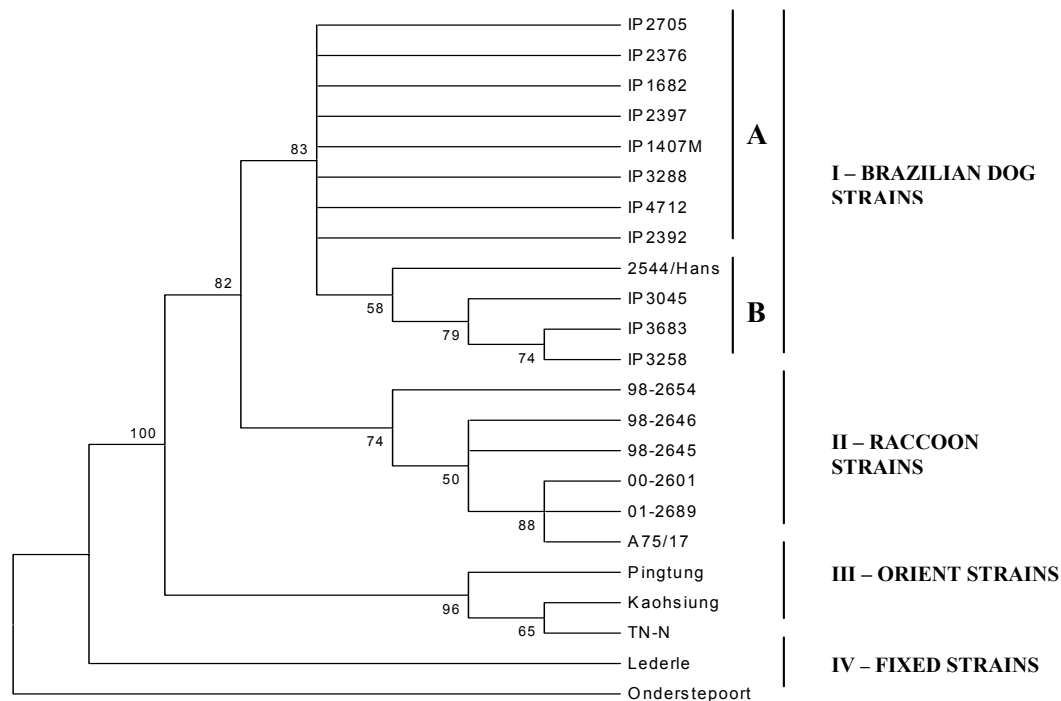


Figure 1. Neighbor-joining phylogenetic tree based on partial sequences of the 5' half of the CDV N gene. Cluster I: Brazilian dog strains; cluster II: raccoon strains; cluster III: Orient strains and cluster IV: fixed strains. Numbers above each node are 1,000 bootstrap replicate values.

The N gene has approximately 1,683 nucleotides with highly conserved regions in the first 2/3 of the gene among members of the genus *Morbillivirus* (Parks et al., 1992; Curran et al., 1993; Frisk et al., 1999), and this characteristic makes the gene a good molecular target for CDV detection and sequencing. However, as described by Yoshida et al. (1998), some degree of variation of the N gene among CDV isolates has been demonstrated, also making this gene suitable for differentiating between strains of CDV and for phylogeny-based molecular epidemiology.

In this study, the 480bp that were sequenced belong to the central region of the N gene, which is the most conserved region (Parks et al., 1992) and thus displays the greatest similarity between samples. Analysis of a conserved area provides sufficient resolution to reach reliable conclusions (Russo, 2001).

Phylogenetic analysis showed that the 11 Brazilian CDV strains segregated into a single cluster (cluster I, bootstrap= 82 ID= 99.2%) separate from the cluster that includes the Onderstepoort and Lederle vaccine strains (cluster IV, bootstrap= 100, ID= 99.75) (Fig. 1 and Table 2). The nucleotide sequences of the 11 Brazilian CDV strains showed IDs of 95.8% and 95.6% when compared with Onderstepoort and Lederle vaccine strains, respectively. Analysis of the N gene of wild-type strains in Japan also indicated separate clusters for the wild-type strains and vaccine strains (Yoshida et al., 1998).

Two other clusters were formed: cluster II (raccoon-strains, bootstrap= 74 and ID= 99.6%) and cluster III (Orient-strains, bootstrap= 96 and ID= 99.6%). This analysis shows that not only cluster IV but also clusters II and III diverge from cluster I. Intracluster IDs (I x II, I x III and I x IV) are shown in Table 2.

As shown in Fig. 1, cluster I can be separated into two clusters, IA and IB (bootstrap= 83 and ID intra-clusters IA x IB= 99.3%). Cluster IB (bootstrap= 58, ID= 99.5%) contains sequences related to the Brazilian strains IP3045, IP3258 and IP 3683 and the German strain 2544/Hans. It could be suggested that there are two lineages of CDV circulating in Brazilian dogs in the area surveyed, one of which (IB) is similar to the 2544/Hans lineage isolated in Germany.

Table 2. Mean identities of the nucleotides in clusters 1-4 and mean values of intracluster identities.

Clusters	Mean identities (ID)
Cluster I	99.2
Cluster II	99.6
Cluster III	99.6
Cluster IV	99.7
Cluster I A	99.2
Cluster I B	99.5
Clusters I A x I B	99.3
Clusters I x II	99.4
Clusters I x III	98.8
Clusters I x IV	95.9
Clusters I x Hans	99.2
Lederle x I	95.6
Onderstepoort x I	95.8

As the N gene is highly conserved in CDV strains and variations were found in the N-gene fragments of the Brazilian samples, it can be inferred that greater polymorphisms can be found in more variable genes such as the H and F genes, which determine the synthesis of the proteins of the same name involved in receptor-binding and induction of neutralizing antibodies (Blixenkrone-Moller et al., 1992).

Although other CDV-related sequences can be found in GenBank, these were not included in this article because the regions of the genome they cover are not the same as those in the samples investigated in this study. All the samples deposited in GenBank to date that could be aligned with the 11 N-gene sequences of Brazilian dogs generated by RT-PCR using the CDV primers described in this article were used. The vast majority of the N-gene sequences deposited in GenBank are small and cover the 3'-terminal of the gene. This area has greater variability and is therefore not suitable for this study, the aim of which was to analyze the most conserved area of the N gene of CDV.

In conclusion, this is the first report of phylogenetic analysis of CDV detected in Brazil, and the method developed in this study proved efficient in the molecular analysis of CDV. The results of this study can be used in conjunction with those of other studies aimed at increasing prevention and control of canine distemper to improve the health of the canine population as well as that of other animals that are susceptible to the disease.

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